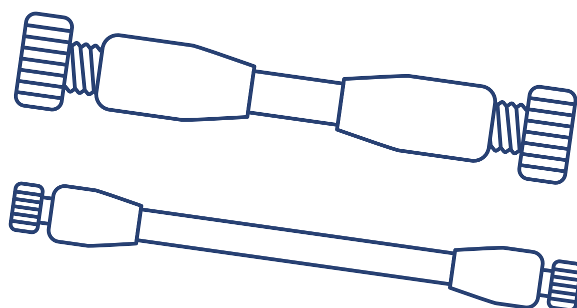

HPLC Column

Technical Guide

GL Sciences

HPLC Column
Technical Guide

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Thank you for choosing GL Sciences' HPLC columns.
GL Sciences' HPLC columns are subjected to a rigorous array of QC tests in our ISO 9001 compliant facility, with special emphasis on the purity of reagents, traceability and consistency of the raw materials, and quality of the final products. To maintain and maximize the performance of GL Sciences' HPLC columns, and to ensure long lifetimes and stability, please refer to this technical guide before use.

1 Specifications

1-1 Recommended Operating Pressures (Maximum Operating Pressures)

GL Sciences' HPLC columns can tolerate backpressures as listed below. Although the columns are packed using the slurry method under high pressures, it is recommended to keep the operating backpressure below the pressures listed below to maintain peak performance and to ensure long column lifetimes and stability.

Recommended Operating Pressures for Analytical Columns

Column Brand	Particle Size	Recommended Maximum Operating Pressures (MPa)
Inertsil, InertSustain, InertSustainSwift, ProteoSil	1.9 µm, 2 µm	80
Inertsil, InertSustain, InertSustain, ProteoSil	3 µm HP	50
Inertsil, InertSustain, InertSustainSwift, ProteoSil	3~10 µm	20*
InertCore Plus C18(2.1 mm I.D.)	2.6 µm	100
InertCore Plus C18(3.0, 4.6 mm I.D.)	2.6 µm	60
InertSphere Sugar-1	5 µm	15
InertSphere Sugar-2, FA-1	9 µm	6
SYRRON AX-1, AX-2	5 µm	12
Capillary EX columns	3 µm, 5 µm	20
Capillary EX Nano columns	3 µm, 5 µm	15

* The maximum operating pressure of the 100 mm I.D. preparative column (Particle Size, 5 µm and 10 µm) is 15 MPa.

Recommended Operating Pressures for Guard Columns

Guard Column	Recommended Maximum Operating Pressures (MPa)
Guard Column for UHPLC	80 (1.9 um) 50 (3 um)
Cartridge Guard Column E Cartridge Guard Column Ei PREP Guard Cartridge	20
Conventional Guard Column Conventional Mini Guard Column Capillary Micro Guard Preparative Guard Column	20
SYPRON AX-1, AX-2 Guard Column	12
InertSphere Sugar-1 Guard Column	15
InertSphere Sugar-2, FA-1 Guard Column	6

1-2 Temperature and pH Range

The recommended operating pH range and temperatures are listed below. To maximize column lifetime, the pH and temperature of the mobile phase should be within the recommended range. Furthermore, the use of aqueous mixture of organic solvent and buffer is recommended as the mobile phase; the use of 100% buffer is NOT recommended. When operating at high pH, lower operating temperatures are recommended for longer column lifetime. Working at high temperatures may also result in shorter column lifetimes. Please note that the column lifetime will vary depending upon the operating temperature, along with the type and concentration of the buffer.

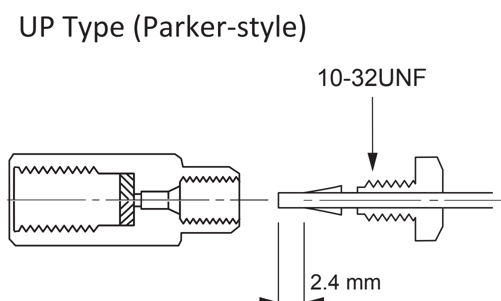
<table>
<tr><th rowspan="2">Column</th><th rowspan="2">Maximum Operating Temperature</th><th colspan="3">pH Range</th></tr>
<tr><th>20–40 ℃</th><th>40–50 ℃</th><th>50–60 ℃</th></tr>
<tr><td>Inertsil series,
ProteoSil 300-C18, 300-C8, 300-C4, 100-SEC, 300-SEC</td><td rowspan="8">60 ℃</td><td colspan="2">2–7.5</td><td>2–7</td></tr>
<tr><td>InertSustain C18, InertSustain AQ-C18
InertSustainBio C18, InertSustainSwift C18
ProteoSil 100-C18, 200-C18</td><td colspan="2">1–10 (*)</td><td>1–7</td></tr>
<tr><td>InertSustain C8, InertSustain Phenylhexyl,
InertSustainSwift C8
ProteoSil 200-C8</td><td>1–10 (*)</td><td>1–9 (*)</td><td>2–7</td></tr>
<tr><td>InertSustain AX-C18</td><td>1–9 (*)</td><td>1–8 (*)</td><td>2–7</td></tr>
<tr><td>InertSustain C30</td><td colspan="2">1–7.5</td><td>1–7</td></tr>
<tr><td>InertSustain Amide
ProteoSil HILIC</td><td colspan="2">2–8.5 (*)</td><td>2–7</td></tr>
<tr><td>InertSustain PFP, InertSustain Phenyl,
InertSustain NH2, InertSustain Cyano</td><td colspan="2">2–7.5</td><td>2–7</td></tr>
<tr><td>InertCorePlus C18</td><td colspan="2">1–10</td><td>1–7</td></tr>
<tr><td>InertSphere Sugar-1</td><td>80 ℃</td><td colspan="3">2–14 (5–80 ℃)</td></tr>
<tr><td>SYPRON AX-1, AX-2</td><td>50 ℃</td><td colspan="2">3–7</td><td>-</td></tr>
<tr><td>InertSphere FA-1, Sugar-2</td><td>95 ℃</td><td colspan="3">1–14 (5–95 ℃)</td></tr>
</table>

* For method development at low pH’s (pH 1–2), the use of trifluoroacetic acid, formic acid, acetic acid, or phosphate buffer is recommended. At high pH’s (pH 10), the buffer concentration should be adjusted to 5 mM using an additive such as trimethylamine.
Lower operating temperatures are recommended for mobile phases with pH’s at the edge of the recommended range (pH 1–2 or pH 9–10).

1-3 Column End-fittings

UP Type (Parker-style) is the standard end-fittings of GL Sciences’ columns.

UP Type (Parker-style)



2 Shipping Solvents

2-1 Shipping Solvents

GL Sciences' HPLC columns are shipped in solvents as listed below. Please note that normal-phase columns are shipped in non-aqueous solvents.

	Columns	Particle Size, Dimensions	Shipping Solvent
Reversed-Phase Columns HILIC Columns	InertSustain Series C18, C8, AQ-C18, AX-C18, C30, PFP Phenylhexyl, Phenyl, Cyano, Amide, AG InertSustainSwift C18, nertSustainSwift C8 InertSustainBio C18 Inertsil Series ODS-4, ODS-4V, ODS-3, ODS-3V, ODS-HL, ODS-SP, ODS-P, ODS-EP, ODS-2, ODS, Amide, HILIC, C8-4, C8-3, C8, C4, Ph-3, Ph, WP300 C18, WP300 C8, WP300 C4, Peptides, Acrolein, Sulfa C18, TMS InertCore Plus C18 MonoSelect C18 for HTS, MonoClad C18-HS ProteoSil Series 100-C18, 200-C18, 200-C8, 300-C18, 300-C8, 300-C4, HILIC	All Particle Sizes & Dimensions	Acetonitrile/Water
Normal-Phase Columns	InertSustain NH2 (*) Inertsil Series NH2 (*), Diol, CN-3, SIL-100A, SIL-150A, WP300 SIL, WP300 Diol ProteoSil 100-SEC, ProteoSIl 300-SEC	All Particle Sizes & Dimensions	*n*-Hexane/Ethanol
Ion-Exchange Columns	Inertsil AX, Inertsil CX	All Particle Sizes & Dimensions	100% Methanol
Polymer Columns	InertSphere Sugar-1	All Particle Sizes & Dimensions	100 mM Sodium Hydroxide Aqueous Solution
	InertSphere Sugar-2	All Particle Sizes & Dimensions	100% Water
	InertSphere FA-1	All Particle Sizes & Dimensions	1% Phosphoric acid solution
	SYPRON AX-1, SYPRON AX-2	All Particle Sizes & Dimensions	Acetonitrile/10 mM Sodium Hydroxide Aqueous Solution

**InertSustain NH2* and Inertsil NH2 are available for those who prefer 100% acetonitrile as the shipping solvent.

2-2 Column Equilibration Prior to Use

Before switching to a different mobile phase system, it is important to understand the compatibility of the solvents to avoid immiscibility or precipitation on the column as well as in the system. Before injecting any samples, thoroughly equilibrate the column with the mobile phase to ensure stable chromatographic performance. For more details, please refer to the following:

●Column Equilibration Procedures for Reversed-Phase Columns

If the mobile phase does not contain any buffers or additives, equilibrate the column and system with the mobile phase for at least 30 minutes. The column may be considered equilibrated once the column backpressure and baseline are steady.

If the mobile phase contains buffers or additives, first equilibrate the column and system with an aqueous organic solvent mixture with the same ratio as that in the final buffered mobile phase. For example, if the final mobile phase is acetonitrile/buffer (20:80), equilibrate the column and system first with acetonitrile/water (20:80) for at least 30 minutes, then with acetonitrile/buffer (20:80) for at least 30 minutes. The column may be considered equilibrated once the column backpressure and baseline are steady.

●Column Equilibration Procedures for Normal-Phase Columns

If the mobile phase does not contain any additives, equilibrate the column and system with the mobile phase for at least 30 minutes. The column may be considered equilibrated once the column backpressure and baseline are steady.

If the mobile phase contains additives, equilibrate the column and system using a solvent mixture with a similar composition as in the final mobile phase but without the additive(s). For example, if the mobile phase is n-hexane/ethanol/acetic acid (900:100:1), first equilibrate the column and system with n-hexane/ethanol (900:100) for at least 30 minutes, then with the final mobile phase for at least 30 minutes. The column may be considered equilibrated once the column backpressure and baseline are steady.

When using normal-phase column for reversed-phase methods, the column must first be properly equilibrated before use. For more details, please refer to Section 2-3.

●Column Equilibration Procedures for HILIC Columns (InertSustain Amide, Inertsil Amide, and Inertsil HILIC)

If the mobile phase does not contain any buffers or additives, equilibrate the column and system with the mobile phase for at least 120 minutes. The column may be considered equilibrated once the column backpressure and baseline are steady.

If the mobile phase contains buffers or additives, equilibrate the column and system with an aqueous organic solvent mixture with the same ratio as in the final buffered mobile phase. For example, if the mobile phase is acetonitrile/ammonium acetate solution (90:10), first equilibrate the column and system using acetonitrile/water (90:10) for at least 30 minutes, then with the final mobile phase for at least 120 minutes. The column may be considered equilibrated once the column backpressure and baseline are steady.

●Column Equilibration Procedures for PEEK and UHPLC PEEK Columns

The column equilibration procedures shall be the same as that for a stainless steel column prior to the analysis. Avoid the use of tetrahydrofuran or chloroform as these solvents can weaken the PEEK hardware causing it to become brittle.

2-3 Column Equilibration Procedures for Normal-Phase Columns to be used under Reversed-Phase Methods

GL Sciences' normal-phase columns can be used under either normal- or reversed-phased conditions. As described in Section 2-1, normal-phase columns are shipped in non-aqueous solvents, and are ready to use under normal-phase conditions. However, if these columns are to be used under reversed-phase separations, please refer to the following procedures before use.

For Inertsil NH2 (5 µm, 150 x 4.6 mm I.D.)

Step 1: Flush the column and system with isopropyl alcohol at 0.5 mL/min for at least 60 minutes.

Step 2: If the final mobile phase contains buffers or additives, first equilibrate the column and system with an aqueous solvent mixture with the same ratio as in the final buffered mobile phase. For example, equilibrate the column and system with acetonitrile/water (90:10) at 0.5 mL/min for at least 60 minutes.

Step 3: Next, equilibrate with acetonitrile/buffer (90:10) at 0.5 mL/min for at least 60 minutes. The column may be considered equilibrated once the column backpressure and baseline are steady.

Step 4: Inject the sample several times to confirm that the retention times are reproducible.

Reproducible results are highly dependent on proper column equilibration. As shown in Figure B, the retention times are reproducible for a column that was properly equilibrated. In contrast, as shown in Figure A, variable retention times were observed for an improperly equilibrated column.

HPLC Conditions

Column : Inertsil NH2
(5 µm, 150 x 4.6 mm I.D.)
Eluent : CH_3CN/H_2O (75:25, v/v)
Flow Rate : 1.0 mL/min
Col. Temp. : 40 °C
Detection : RI

Sample:
1. Fructose
2. Glucose
3. Sucrose
4. Maltose
5. Lactose

Figure A.

The column was equilibrated only with an aqueous organic solvent mixture, with the same ratio as in the final mobile phase. In other words, the column was not initially flushed with isopropyl alcohol.

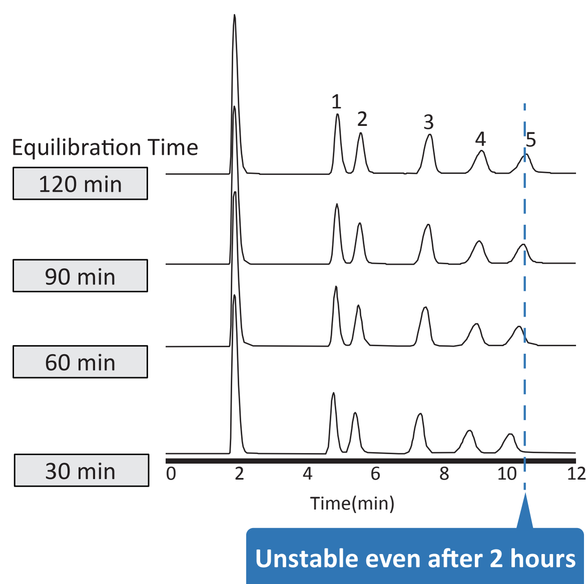
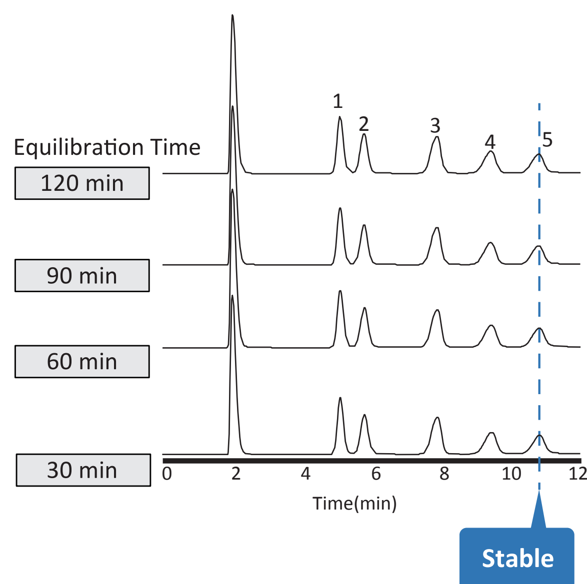


Figure B.

The column was properly equilibrated in accordance with the procedures described in page 7, Section 2-3.



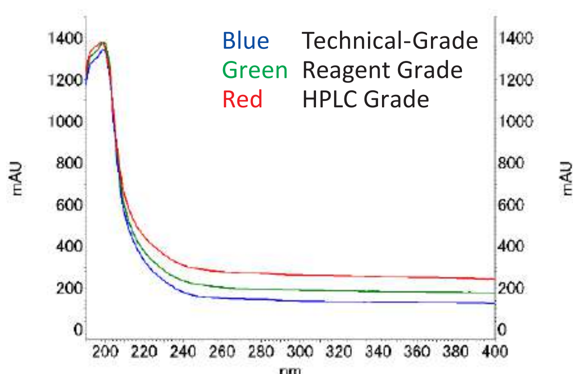
3 Mobile Phase

3-1 Grades of Solvents

To maximize column performance, use high-quality HPLC or MS-grade solvents. Lower grade solvents may contain suspended particulates that will clog the inlet frit of the column, resulting in higher backpressures. Furthermore, lower grade solvents or reagents may include contaminants/ impurities or additives (stabilizing agents) that may absorb UV (ultraviolet) and interfere the chromatographic results. Select the highest grade of solvent and reagents in accordance with your method and application.

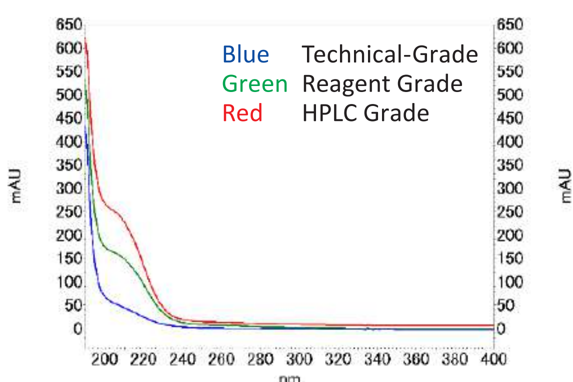
The UV absorbance curves various grades of solvents of is shown below.

●Methanol



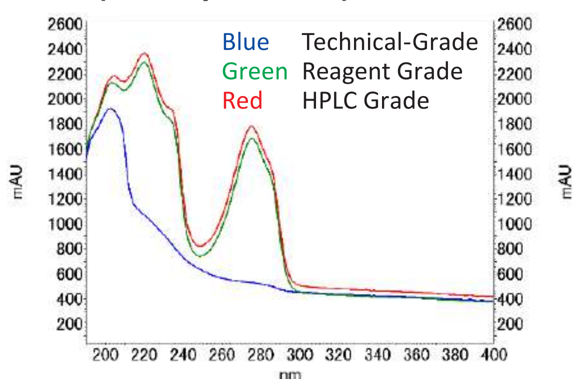
Significant differences in UV absorbance were not observed for methanol.

●Acetonitrile



Lower grades acetonitrile (red and bed lines) exhibit UV absorbance at lower wavelengths.

●THF (Tetrahydrofuran)



When using THF, ensure that it is HPLC-grade. Lower grades of THF (red and green lines) may contain antioxidants that can absorb UV and therefore affect the baseline. Additionally, old bottles of THF may exhibit high UV background due to the formation of peroxides.

3-2 Degassing Mobile Phase Solvents

When solvents are exposed to the atmosphere, air gradually dissolves into the solvent. The amount of dissolved air depends on the composition of the mobile phase, temperature, and pressure. When the aqueous component and the organic solvents are mixed, each contribute to the total content of dissolved air in the mixture. For both isocratic and gradient mobile phases, the amounts of dissolved gas are proportional to their relative solvent volumes. However, if the solubility of air in the mixtures is less than those of the individual components, the mixture is supersaturated with air, and is susceptible to the formation of air bubbles that may cause the following problems:

●Possible Problems Caused by Insufficient Degassing of Solvents

Places where air bubbles may have formed	Problematic Symptoms	Estimated Chromatographic Results
Pump	Unstable or fluctuating flow of the mobile phase	Shift in retention times. Change in peak areas.
Column	Decrease in column efficiency.	Distorted peak shapes. Decreased column efficiency.
Detector	Interference on the detection of analyte.	Baseline drift or baseline noise. Decreased sensitivity.

●Degassing Methods

The following table lists the features of various degassing methods:

Methods	Operation Procedures	Features
Vacuum	Attach an aspirator to the mobile phase bottle and degas by applying vacuum for at least 15 minutes with agitation to remove air bubbles.	Although a low-cost solution, the solvent composition may change due to evaporation.
Sonication	Place the mobile phase bottle into an ultrasonic cleaner and sonicate for at least 10 minutes.	Safe and easy, but air bubbles cannot be completely removed.
Vacuum + Sonication	Place the mobile phase bottle in an ultrasonic cleaner. Attach an aspirator and apply vacuum for at least 2 minutes with sonication.	Solvents can be degassed in a short time, however, the solvent composition can quickly change.
Helium Sparge	Apply a stream of helium through the mobile phase to sweep out dissolved air.	Highly effective in reducing the dissolved air in common solvents to levels below the saturation level of mixtures. However, the cost of helium gas is high.
Degasser	A membrane-based degasser uses a tube of semi-permeable membrane passing through a vacuum chamber. Gases diffuse through the membrane whereas the solvents are retained within the tube.	Easy-to-use and the solvent content does not change significantly.

●Cautions on Degassing

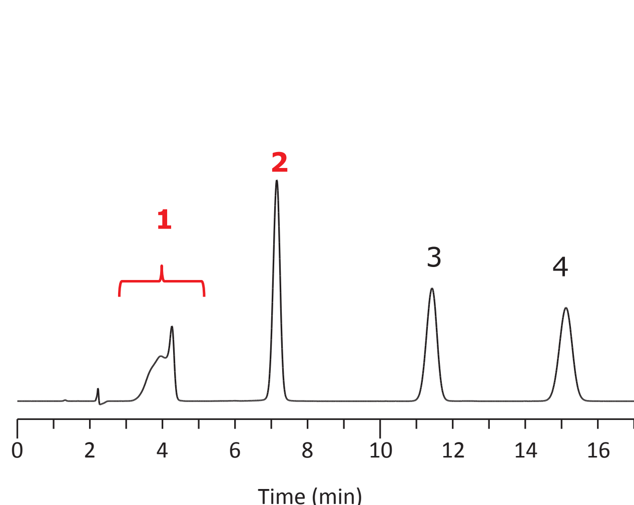
Because low temperatures increase gas solubility, solvents stored under cold conditions may contain large amounts of dissolved air. In the case of acetonitrile/water mixtures, the temperature decreases after mixing these two solvents, thus introducing additional dissolved air into the mixture. Solvents that are stored under cold conditions should be warmed to room temperatures then fully degassed prior to their use.

4 Sample Diluent

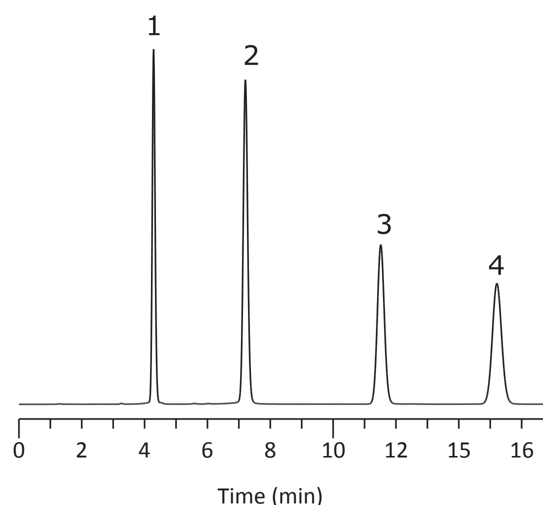
4-1 Effect of Different Elution Strengths between the Sample Diluent and Mobile Phase

Ideally, the sample diluent should have a composition similar to or identical to that of the mobile phase. As illustrated below, using a sample diluent that is stronger than the mobile phase can lead to distorted peak shapes, such as peak splitting.

Sample Diluent: 100% Acetonitrile



Sample Diluent: Mobile Phase



HPLC Conditions

Column : InertSustain AQ-C18 (5 µm, 150 x 4.6 mm I.D.)
Eluent : CH_3CN/H_2O (10:90, v/v)
Col. Temp. : 40 °C
Detection : UV 280 nm
Injection Volume : 10 µL

Sample:
1. 5-Hydroxymethyl-2-furaldehyde
2. 2-Furfural
3. 2-Acetylfuran.
4. 5-Methyl-2-furfural

4-2 Effect of Different pH values between the Sample Diluent and Mobile Phase

Problems with peak shape, such as peak splitting or fronting, may be observed when the pH of the sample diluent and that of mobile phase are significantly different. Such problem can be overcome by diluting the sample solution with the mobile phase or decreasing the injection volume.

5 Column Cleaning and Storage

Residual ion-pairing reagents, acids, or buffer salts in the column will promote the hydrolysis of the bonded phase resulting in shorter column lifetimes. Furthermore, extended storage in highly aqueous mobile phases may cause sample matrices such as lipids to accumulate in the column resulting in distorted peak shapes or decreased column efficiency. To prolong column lifetimes, diligently clean the column after every analysis.

5-1 Cleaning Reversed-Phase Columns

●If the mobile phase does not contain any buffers or ion-pairing reagents

Use high concentrations of organic solvents to remove lipophilic contaminants. Increase the ratio of organic solvents up to 100%, then flush the column using 5 column volumes.
If excessive backpressures are observed, reduce and adjust the flow rate.

Example Column Dimensions:	4.6 mm I.D. x 250 mm
Flow Rate:	1 mL/min
Mobile Phase:	acetonitrile/water (65:35)

Step 1: Clean the column with 100% acetonitrile at 1 mL/min for at least 30 minutes.

●If the mobile phase contains buffers

First, flush the column with an aqueous organic solvent, using the same ratio as in the final buffered mobile phase. For example, first clean the column with 20% acetonitrile in water for at least 30 minutes, followed by 100% acetonitrile.

Example Column Dimensions:	4.6 mm I.D. x 250 mm
Flow Rate:	1 mL/min
Mobile Phase:	10 mM KH_2PO_4/acetonitrile (80:20)

Step 1: Clean the column with acetonitrile/water (20:80) at 1 mL/min for at least 30 minutes.
Step 2: Clean the column with 100% acetonitrile at 1 mL/min for at least 30 minutes.

*When re-using the column , follow the procedures below to avoid possible precipitation of the mobile phase buffers within the column.

Step 1: Equilibrate the column with acetonitrile/water (20:80) at 1 mL/min for at least 30 minutes.
Step 2: Equilibrate the column with the buffered mobile phase at 1 mL/min for at least 30 minutes.
Step 3: The column may be considered fully equilibrated once the backpressure and baseline are steady.

●If the mobile phase contains ion-pairing reagents

Depending on the ion-pairing reagent type, precipitation may occur when cleaning the column with 100% water. First, clean the column with an aqueous organic solvent mixture with the same ratio as in the final mobile phase containing an ion-pairing reagent. For example, wash the column with acetonitrile/water (10:90) for at least 30 minutes, followed by acetonitrile/water (50:50) for at least another 30 minutes. Higher ratios of the organic solvent should be used to remove ion-pairing reagents containing long alkyl chains.

Example Column Dimensions	4.6 mm I.D. x 250 mm
Flow Rate	1 mL/min
Mobile Phase	10 mM KH_2PO_4 + 2 mM IPCC-09* (pH 2.5)/acetonitrile (90:10)

Step 1: Clean the column with acetonitrile/water (10:90) at 1 mL/min for at least 30 minutes.
Step 2: Clean the column with acetonitrile/water (50:50) for at least 30 minutes.

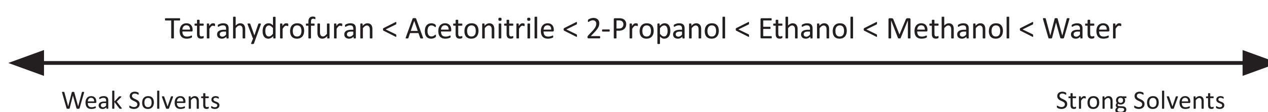
* IPCC-09: Sodium 1-nonanesulfonate
* Please be aware that the complete removal the ion-pairing reagent may not be possible.
Because ion-pairing reagents can alter column selectivity, it is strongly recommended to dedicate columns to ion-pairing methods to avoid problems with reproducibility.

●When strong ionic components are analyzed with InertSustain AX-C18

Clean the column with a methanol solution containing 50 - 100 mM ammonium acetate.

5-2 Cleaning HILIC Columns

Under HILIC mode, polar analytes are retained with high organic mobile phases. The following describes the relative elution strengths of solvents under HILIC mode.



●Cleaning InertSustain Amide Columns

To avoid the precipitation of mobile phase buffers, clean the column with an aqueous organic solvent mixture, using the same ratio as in the final buffered mobile phase. Next clean the column with acetonitrile/water (50:50) to remove highly polar contaminants.
If the column still exhibits shifts in retention times or distorted peak shapes, wash the column with 100% water for at least 30 minutes. After cleaning the column, thoroughly equilibrate the column with the final mobile phase prior to use. Store the InertSustain Amide column in 100% acetonitrile.

Example Column Dimensions:	4.6 mm I.D. x 250 mm
Flow Rate:	1 mL/min
Mobile Phase:	5 mM CH_3COONH_4/acetonitrile (10:90)

Step 1: Clean the column with acetonitrile/water (90:10) at 1 mL/min for at least 30 minutes.
Step 2: Clean the column with acetonitrile/water (50:50) at 1 mL/min for at least 30 minutes.

●Cleaning Inertsil HILIC Columns

To avoid the precipiation of the mobile phase buffers, clean the column with an aqueous organic solvent mixture, using the same ratio as in the buffered mobile phase. Clean the column with 100% water to remove highly polar contaminants.

Example Column Dimensions:	4.6 mm I.D. x 250 mm
Flow Rate:	1 mL/min
Mobile Phase:	5 mM CH_3COONH_4/acetonitrile (10:90)

Step 1: Clean the column with acetonitrile/water (90:10) at 1 mL/min for at least 30 minutes.
Step 2: Clean the column with 100 % water at 1 mL/min for at least 30 minutes.

●Cleaning InertSustain NH2 Columns

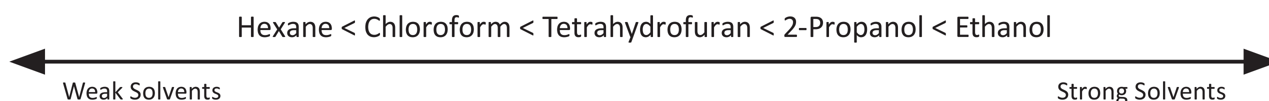
To avoid the precipitation of the mobile phase buffers, clean the column with an aqueous organic solvent mixture, using the same ratio as in the final buffered mobile phase. Next, clean the column with acetonitrile/water (50:50) to remove highly polar contaminants.
If the column still exhibits shifts in retention times or distorted peak shapes, clean the column with 50 mM ammonium formate (or ammonium acetate)/acetonitrile (50:50) for at least 30 minutes. After cleaning the column, equilibrate the column with the final mobile phase prior to use. Store the InertSustain NH2 column in 100% acetonitrile.

Example Column Dimensions:	4.6 mm I.D. x 250 mm
Flow Rate:	1 mL/min
Mobile Phase:	5 mM CH_3COONH_4/acetonitrile (10:90)

Step 1: Clean the column with acetonitrile/water (90:10) at 1 mL/min for at least 30 minutes.
Step 2: Clean the column with acetonitrile/water (50:50) at 1 mL/min for at least 30 minutes.

5-3 Cleaning Normal-Phase Columns

Normal-phase separations depend upon polar interactions that are defined by the bonded phase, and the non-polar mobile phase. Therefore, polar analytes are strongly retained whereas non-polar analytes are weakly retained. Clean the column with polar solvents to remove highly polar contaminants.
The following describes the relative elution strength of solvents used in normal-phase mode.



●Cleaning Inertsil SIL-100A, Inertsil SIL-150A, Inertsil WP300 SIL, Inertsil NH2, Inertsil CN-3, Inertsil Diol, and InertSustain NH2 Columns

Clean the column using ethanol or 2-propanol. Because alcohol solvents are quite viscous, adjust the flow rate to avoid excessive column backpressures.

Example Column Dimension:	4.6 mm I.D. x 250 mm
Flow Rate:	1 mL/min
Mobile Phase:	n-Hexane/2-Propanol/Acetic acid (90:10:0.1)

Step 1: Clean the column with 100% 2-propanol at 0.2 mL/min for at least 60 minutes.

5-4 Cleaning Ion-Exchange Columns

To efficiently remove both ionic and hydrophobic compounds, two solvents are used to clean the column. First, remove ionic compounds using a mobile phase with increased concentration of the buffer (maximum 200 mM for silica-based columns). Next, remove the hydrophobic compounds using a highly organic solution. It is recommended to flush the column with an intermediate solvent between each cleaning step to avoid salt precipitation. Some polymer-based columns are not compatible with highly organic solutions - please check the compatibility beforehand.

(Example 1)	
Example Column:	Inertsil CX 4.6 mm I.D. x 250 mm
Flow Rate:	1 mL/min
Mobile Phase:	20 mM $HCOONH_4$ (pH 5.0, HCOOH)

Step 1: Clean the column with 100% deionized water at 1 mL/min for at least 30 minutes.
Step 2: Clean the column with 200 mM $HCOONH_4$ (pH 5.0, HCOOH) at 1 mL/min for at least 30 minutes.
Step 3: Clean the column with 100% deionized water at 1 mL/min for at least 30 minutes.
Step 4: Clean the column with 100% acetonitrile at 1 mL/min for at least 30 minutes.

Note: To avoid salt precipitation, flush with 100% deionized water before reusing the column.

(Example 2)	
Example Column:	Inertsil AX 4.6 mm I.D. x 250 mm
Flow Rate:	1 mL/min
Mobile Phase:	KH2PO4, H3PO4

Step 1: Clean the column with 100% deionized water at 1 mL/min for at least 30 minutes.
Step 2: Clean the column with 60 mM KH_2PO_4 (pH 3.0, H_3PO_4) at 1 mL/min for at least 30 minutes.
Step 3: Clean the column with 100% deionized water at 1 mL/min for at least 30 minutes.
Step 4: Clean the column with 100% acetonitrile at 1 mL/min for at least 30 minutes.

Note: To avoid salt precipitation, flush with 100% deionized water before reusing the column.

(Example 3)	
Example Column:	Sypron AX-1/AX-2 2.1 mm I.D. x 150 mm
Flow Rate	0.1 mL/min
Mobile Phase	100 mM CH_3COONH_4 in H_2O/acetonitrile (70:30)

Step 1: Clean the column with 10 mM NaCl aqueous solution/acetonitrile (80:20) at 0.1 mL/min for at least 60 minutes.
Step 2: Clean the column with 100 mM NaCl aqueous solution at 0.1 mL/min for at least 6 hours. Adjust the flow rate to avoid excessive column backpressures.
Step 3: Clean the column with acetonitrile/deionized water (50:50) at 0.1 mL/min for at least 2 hours.

Note: Do not connect the column to the detector during these cleaning steps.

(Example 4)	
Example Column:	InertSphere Sugar-1 4.6 mm I.D. x 150 mm
Flow Rate:	0.5 mL/min
Mobile Phase:	100 mM NaOH

Step 1: Clean the column with 1 M NaOH aqueous solution at 0.5 mL/min for at least 60 minutes.
Step 2: Clean the column with eluent at 0.5 mL/min for at least 2 hours.

To remove hydrophobic contaminants, include the following steps between Steps 1 and 2.
Step 1: Clean the column with deionized water at 0.5 mL/min for at least 20 minutes.
Step 2: Clean the column with 100% methanol at 0.5 mL/min for at least 60 minutes.
Step 3: Clean the column with deionized water at 0.5 mL/min for at least 20 minutes.

Note: Do not connect the column to the detector during these cleaning steps.

5-5 Column Storage

Residual ion-pairing reagents, acids, or salts in the column may cause the hydrolysis of the bonded phase resulting in shorter column lifetimes. Furthermore, columns stored under water-rich conditions for extended periods of time may exhibit deteriorated peak shapes or decreased column efficiencies as sample matrices such as lipids can accumulate in the column. To prolong column lifetimes, diligently clean the column after every analysis.
After properly cleaning the column (refer to Sections 5-1 to 5-3), store the column in accordance to the following table.

Bonded Phase	Storage from 1 ~ 10 Days	Storage longer than a few weeks
C18(ODS), C8, C4, C30, Ph, HILIC, Amide	Mobile Phase (without salts, additives)	100 % Acetonitrile
CN, NH2, Diol, SIL, 100-SEC, 300-SEC	Mobile Phase (without salts, additives)	100 % n-Hexane
Inertsil AX, CX	Mobile Phase (without salts, additives)	100 % Acetonitrile
Sugar-1	0.1 M NaOH	
Sugar-2	100% Water	
Sypron AX-1, AX-2	10 mM NaCl aqueous solution/Acetonitrile = 80/20	

*Columns should be stored in a cool and dark place.
*Columns stored for an extended periods should be cleaned then equilibrated prior to use.

6 Tips to Increase HPLC Column Lifetime

To increase column lifetimes, please follow these important guidelines:

6-1 Column Handling

- To avoid deterioration of column performance, do not drop or bump/shock the columns. Disconnect the column from the system after confirming the display of the pressure gauge shows a zero "0" value. Avoid rapid pressure fluctuations to extend column lifetime.

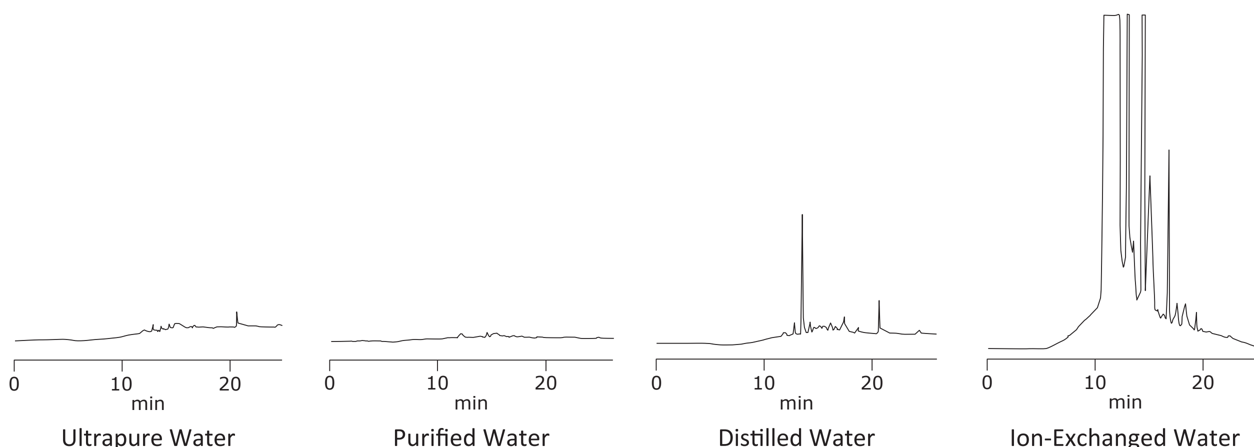
- Use a pump equipped with an inline filter to prevent particulates from worn pump seals or contaminants from the mobile phases from entering the column.

- To maximize column lifetimes, use the column within the operating conditions described in Section 1. Please note that operating under extreme pHs, temperatures, and backpressures will result in shorter column lifetimes.

- Residual ion-pairing reagents, acids, or salts in the column may cause the hydrolysis of the bonded phase resulting in shorter column lifetimes. To prolong column lifetimes, diligently clean the column after every analysis. For more details, please refer to Section 5.

- Only use ultrapure water in the mobile phase. Routinely inspect the water purification system to ensure its proper performance. Usage of contaminated water may result in noisy or drifting baselines and/or ghost peaks that appear under gradient conditions. Additionally, use freshly treated purified water (or aqueous solvents), and avoid prolonged storage. Ultrapure water may absorb contaminants from the laboratory atmosphere and/or containers.

- Comparison of residual contaminants in water among various water purification processes:



6-2 Guard Columns

Guard columns are connected between the sample injector and the column to protect against any contaminants or strongly retained compounds. GL Sciences offer two types of guard columns: 1) a cartridge-type requiring an exclusive holder, and 2) a packed guard column type, packed with the same material as in the analytical column.

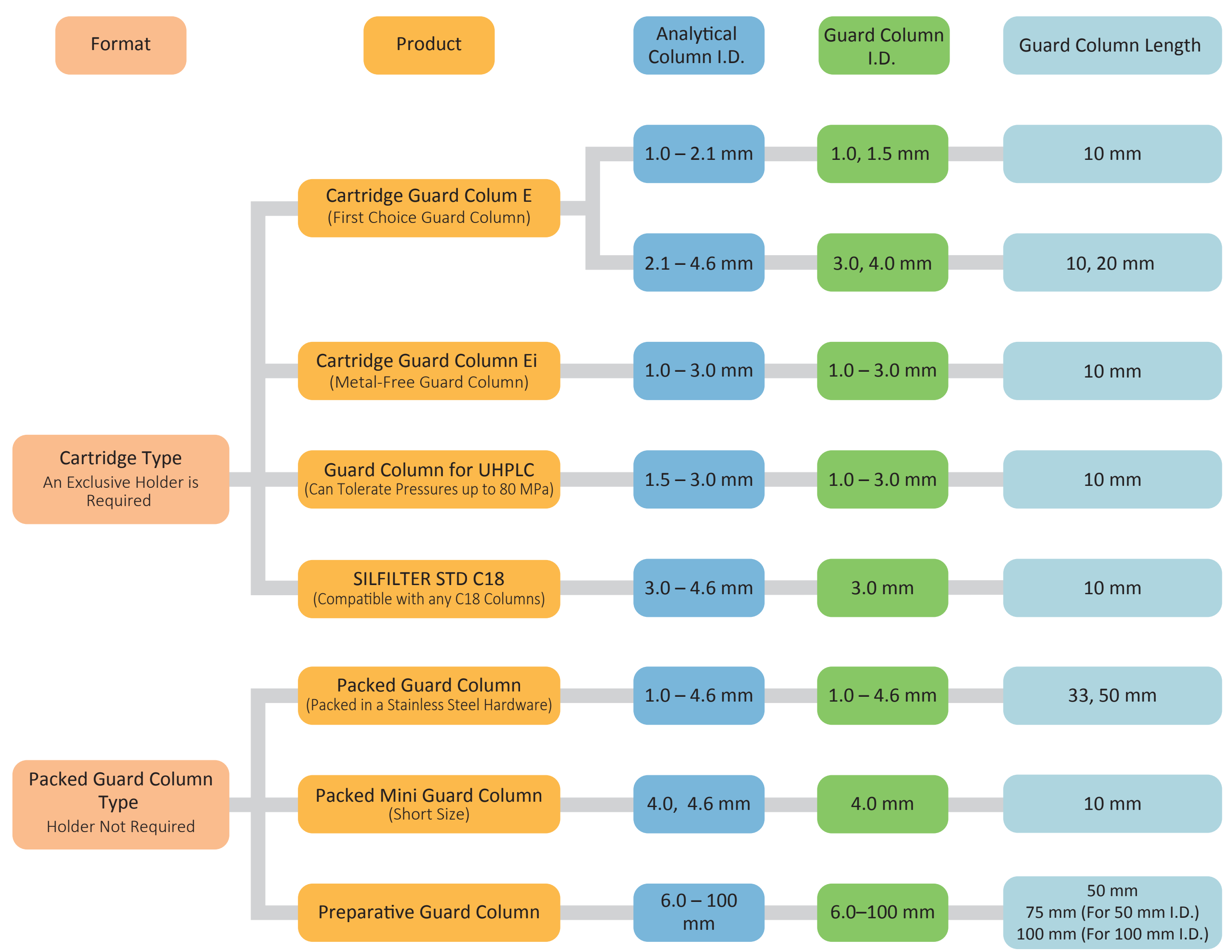
●Selecting the Appropriate Column Protection System

It is best to choose a guard column that contains the same packing material as that in the analytical column. SILFILTER STD C18 is a monolithic silica type guard cartridge, and is compatible with any C18 (ODS) analytical columns.

●Selecting the Appropriate Guard Column Size

Particle Size: Select a guard column that contains the same packing material and particle size as that in the analytical column.

Internal Diameter: Select a guard column with the same I.D. or similar as that for the analytical column.



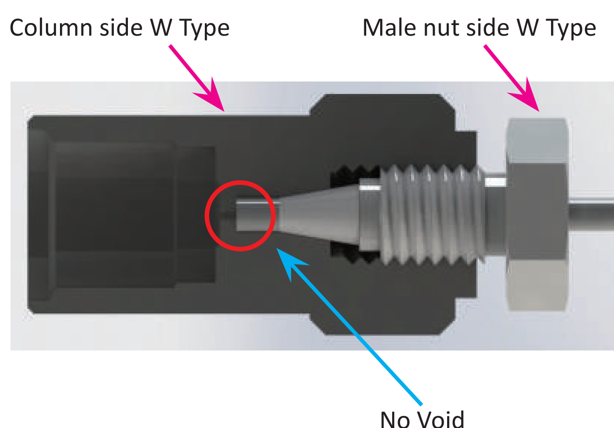
●Guard Column Installation

Column connectors and tubing are required to connect the guard columns to the analytical columns. Select the appropriate connectors for the type of the end-fittings used on the analytical column. Mismatched connector will negatively impact the chromatography due to unnecessary dead volumes.

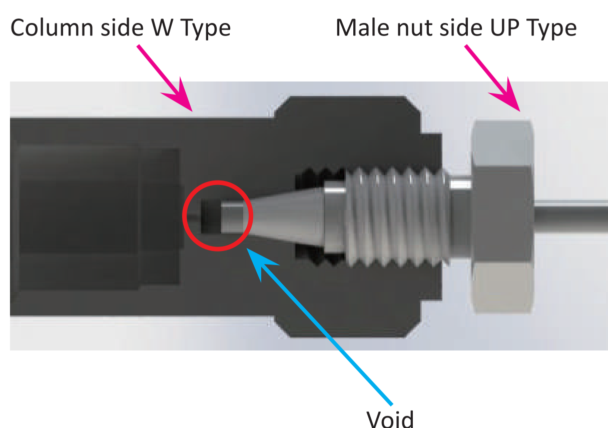
●Type of End-fittings

End-fitting types differ among various manufacturers with different seating depths. Select the appropriate end-fittings, otherwise, chromatographic performance will be negatively impacted due to unnecessary dead volumes. For more details on the end-fitting types, please refer to Section 1-3.

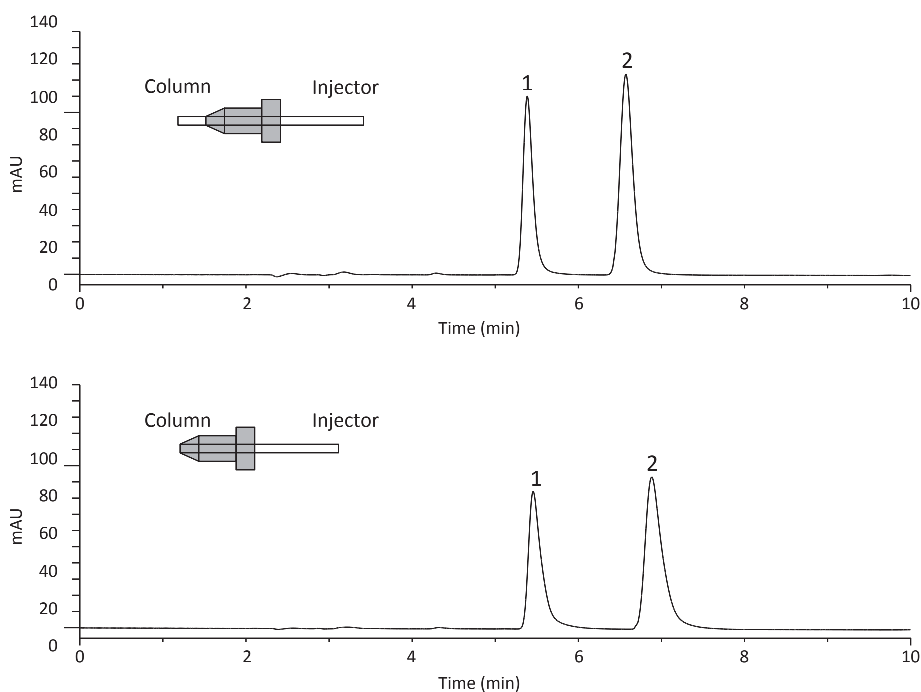
●Proper Column Connection



●Improper Column Connection



●Influence of Extra Dead Volume on Chromatographic Performance



6-3 Sample Preparation

●Sample Filtration

Particulates from various sources contribute to column contamination. To maximize column lifetimes, filter all samples through a disposable syringe filter to remove particulates.

●Sample Preparation for Biological Samples

Proteins are present in biological samples that can interfere with the separation and quantitation of the target analytes. Additionally, the protein matrices can reduce column lifetimes, especially under reversed-phase conditions. In some cases, target analytes may bond to the protein matrices to negatively impact the accuracy, precision, and robustness of the method.

●Deproteinization Methods

Protein Precipitation

Protein removal can be accomplished by first adding organic solvent to the biological sample to denature the protein matrices. Centrifuge the precipitated sample solution, then use the supernatant after passing through a filter.

Ultrafiltration

In this case, the primary basis for the removal of proteins is based upon molecular sizes. Proteins, which are larger than the membrane pores, will be retained at the membrane surface.

Protein Hydrolysis

Protein hydrolysis is the breakdown of protein into smaller peptides and free amino acids using enzymes.

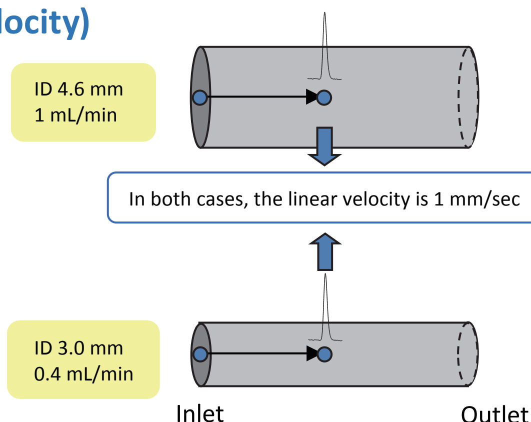
7 Benefits of Scaling Down Column Internal Diameter Size

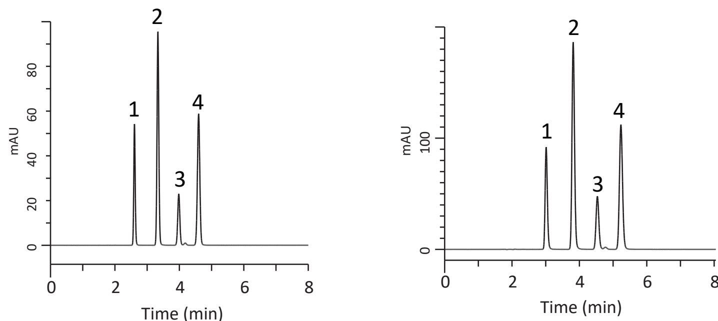
By scaling down to a narrower internal diameter column, the flow rate can be reduced (while maintaining linear velocity), thus achieving cost-savings by reducing solvent consumption. Depending on the detector type, increased sensitivity can also be achieved.
However, caution needs to be taken when scaling down columns – the following adjustments are required to maximize column performance upon scale-down:

7-1 Adjust the Flow Rate (Maintain Linear Velocity)

To maintain equivalent separation and retention times when scaling down a method, it is important to maintain the linear velocity between the original and new methods.
The linear velocity can be calculated using the flow rate and internal diameter of the column.

$$\text{Linear Velocity (mm/s)} = \frac{\text{Flow Rate (mm}^3\text{/s)}}{\text{Column Cross-Section (mm}^2\text{)}}$$





ID 4.6 mm
Flow Rate 1.0 mL/min

ID 3.0 mm
Flow Rate 0.4 mL/min

60% Less Solvent Consumption

1 — Ratio of the Column's Cross-Section — 0.4

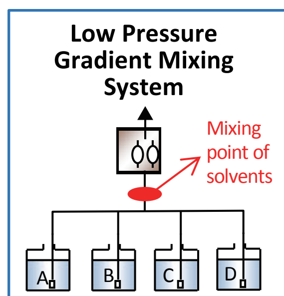
HPLC Conditions

System:	GL7700 HPLC
Column:	InertSustainSwift C18 (3 µm, 150 mm)
Eluent:	CH_3CN/H_2O (65:35,v/v)
Column Temp.:	40 °C
Detection:	UV 254 nm
Injection Vol.:	2.0 µL

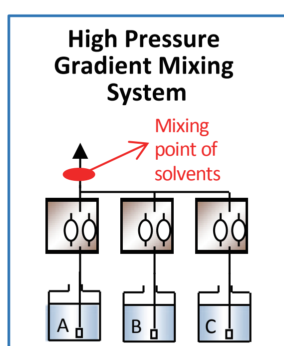
1. Acetophenone
2. Benzene
3. Toluene
4. Naphthalene

7-2 Gradient Delay

A low-pressure gradient mixing system (quaternary pump) employs one pump that delivers the mobile phase to the system. Caution needs to be taken when scaling down the internal diameter.



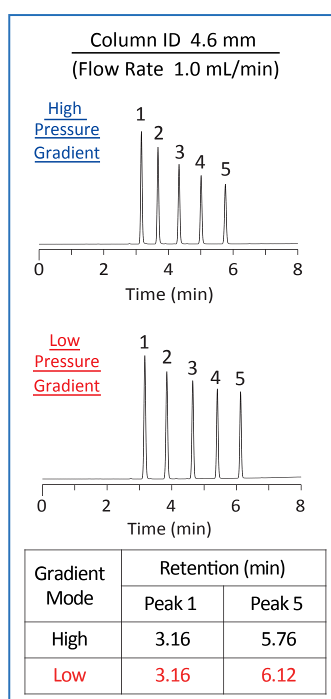
The mixing of the solvents occurs right before the pump unit. As the solvents travel to the pump, the plunger draws the solvents into the pump head(s) and creates a turbulent environment to form the mobile phase mixture. Because the mobile phase is not under pressure at the mixing point, quaternary pump is considered as a low-pressure gradient mixing system.



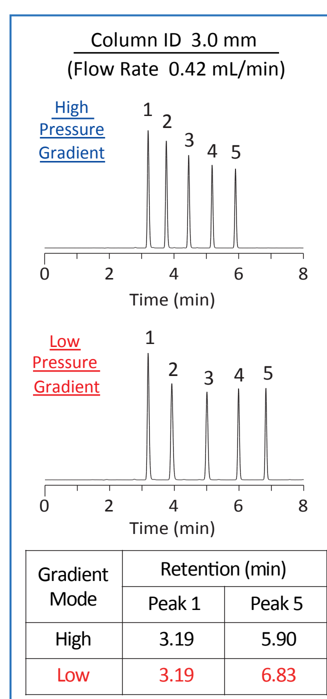
In a high-pressure gradient mixing system, one solvent reservoir is assigned per pump, to provide precise flow for each solvent. The solvents are then combined in a mixing chamber that is located after the pumps. This involves a high-pressure proportioning environment because the solvents are under pressure when they arrive at the mixing point.

●Variation in Gradient Delay Volumes between Low/High-Pressure Gradient Systems

The biggest difference between the low- and high-pressure gradient systems is the gradient delay volume, specifically in the mixer and the tubings. Gradient delay volume is defined as the volume between the point where the mobile phase begins to mix and the inlet to the column. Generally, a low-pressure gradient system has a larger gradient delay volume as the mixing of the mobile phase begins before the pump unit. As shown below, when using narrower ID columns at low flow rates, the gradient delay becomes more prominent on the low-pressure gradient system.



Gradient Mode	Retention (min)	
	Peak 1	Peak 5
High	3.16	5.76
Low	3.16	6.12



Gradient Mode	Retention (min)	
	Peak 1	Peak 5
High	3.19	5.90
Low	3.19	6.83

HPLC Conditions

Column:	InertSustainSwift C18
Standard:	3 µm, 150 x 4.6 mm I.D.
Semi-micro:	3 µm, 150 x 3.0 mm I.D.
Eluent:	A) CH_3CN
	B) H_2O
	A/B = 50:50 - 6min – 100:0,v/v
Column Temp.:	40 °C
Detection:	PDA 270 nm
Injection Volume:	0.5 µL
Mixer Volume:	The volume of solvent delivered in 1 minute
	Standard approx. 1.0 mL
	Semi-micro approx. 0.4 mL

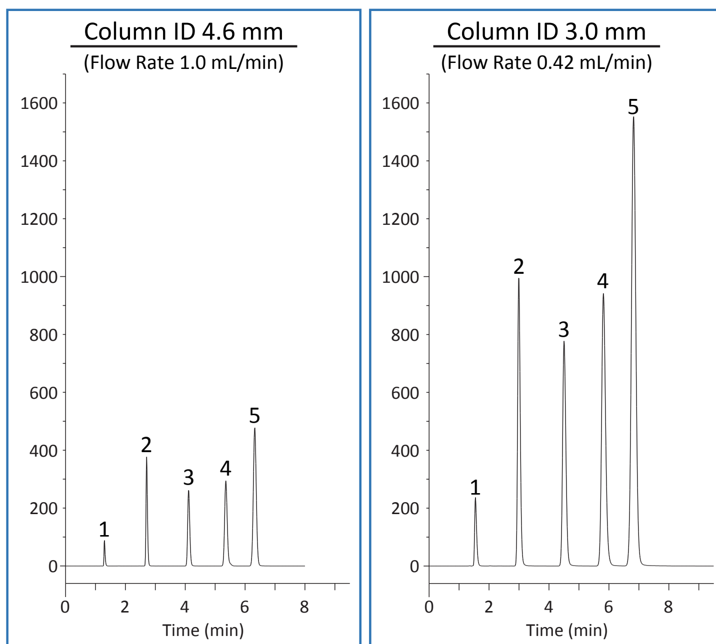
Sample:
1. 4-Methylphenol
2. 4-Ethylphenol
3. 4-Propylphenol
4. 4-Butylphenol
5. 4-Pentylphenol

7-3 Adjusting the Sample Injection Volume

It is recommended to adjust the sample injection volume when scaling down to a narrower ID column.

●Higher Sensitivities

If the injection volume are equal, higher sensitivities can be achieved using a concentration-dependent UV detector.



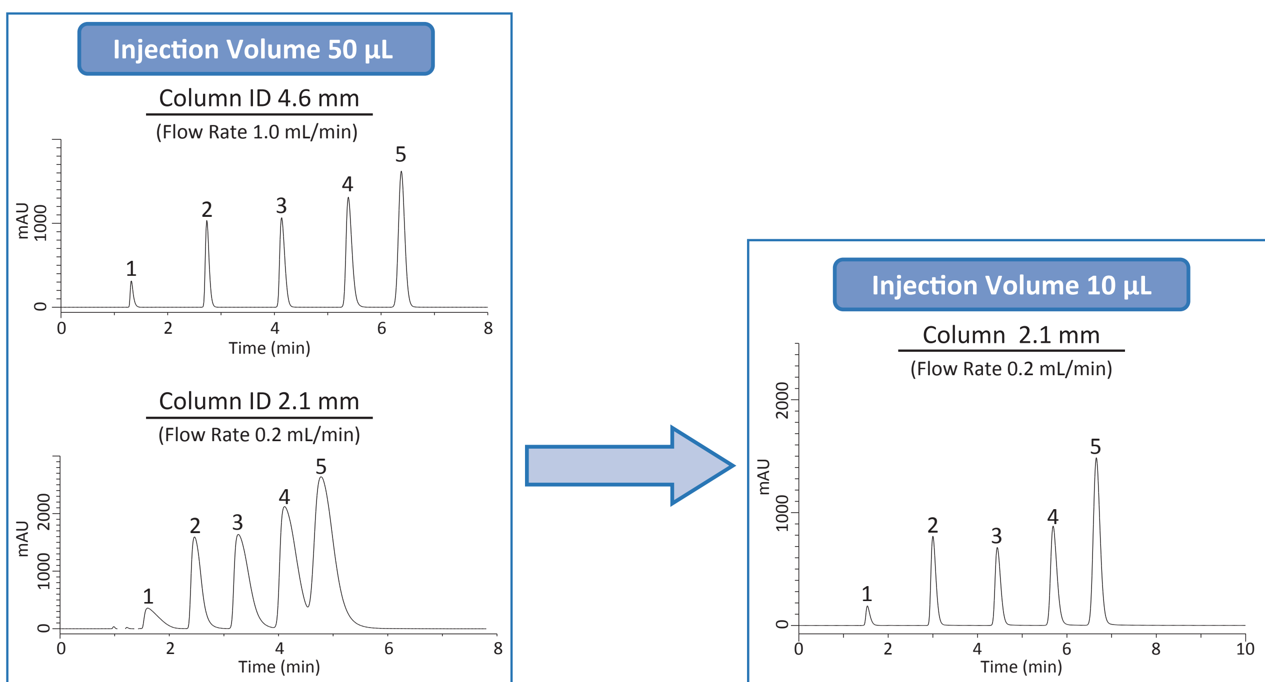
HPLC Conditions

Column:	InertSustain C18
Standard:	3 µm, 150 x 4.6 mm I.D.
Semi-micro:	3 µm, 150 x 3.0 mm I.D.
Eluent:	CH_3CN/H_2O (65:35, v/v)
Column Temp.:	40 °C
Detection:	UV 254 nm
Injection Volume:	10 µL

Sample:
1. Uracil
2. Acetophenone
3. Benzene
4. Toluene
5. Naphthalene

●Poor Peak Shapes

When scaling down the column to a narrower internal diameter, but with the same column length, the sample loading volume should be proportional to the column's cross-section. Accordingly, deteriorated peak shapes may be observed when the polarities of mobile phase and the injection solvent are significantly different. To remedy, the injection volume should be reduced proportionally to the flow rate or the sample solution should be diluted with the mobile phase.

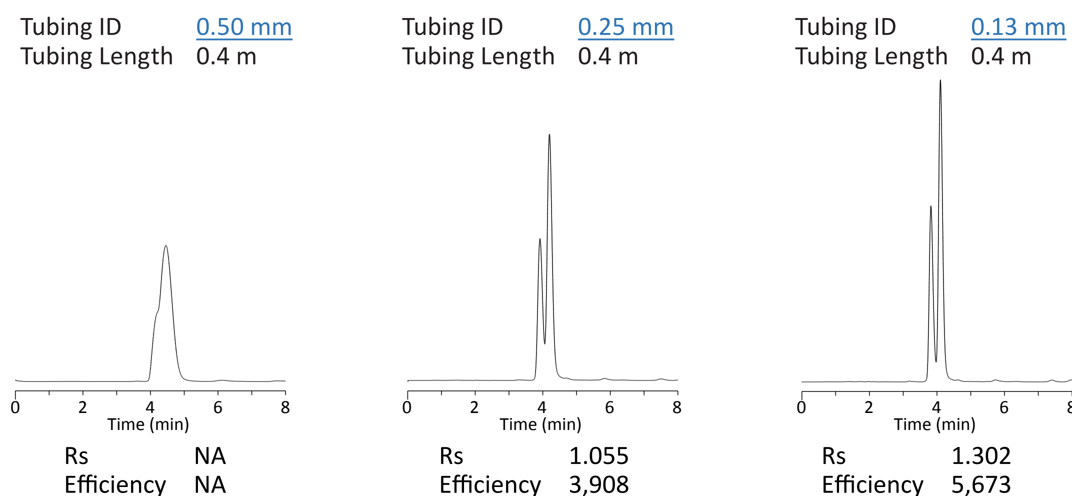


7-4 HPLC Tubing

It is necessary to select the appropriate tubing the HPLC system depending on the internal diameter of the analytical column and the flow rate of the mobile phase. Extra-column volumes due to the tubing between the column outlet and detector inlet can influence band spreading, which will affect the peak shape and efficiency. It is recommended to select the narrowest tubing to minimize band spreading, but not too narrow as it can generate higher backpressures.

●Effect of Extra-Column Volume on Chromatographic Separation

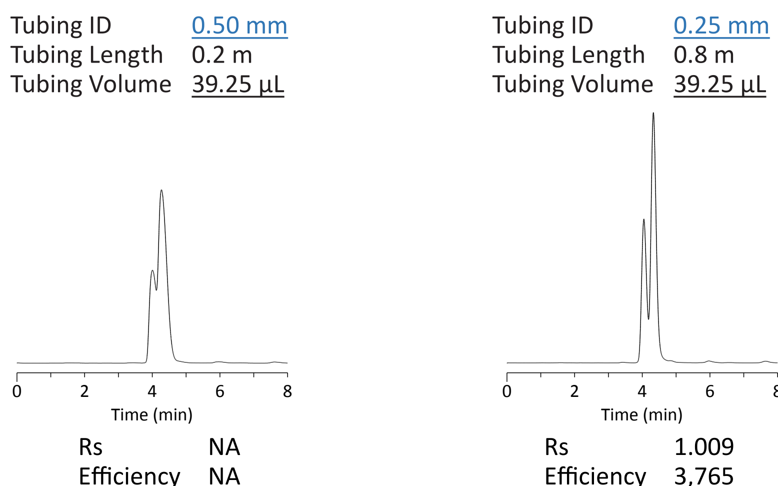
As shown below, extraneous extra-column volume will negatively impact the chromatographic separation. Caution needs to be taken especially when using semi-micro HPLC systems.



HPLC Conditions

Column: InertSustain C18 HP (3 µm, 150 × 2.1 mm I.D.)
Eluent: CH_3CN/H_2O (50:50, v/v)
Flow Rate: 0.2 mL/min
Column Temp.: 40 °C
Detection: UV 254 nm
Sample: o, p-Cresol

As shown below, the use of smaller internal diameters tubings delivers better peak shapes, even with the same total tubing volume.

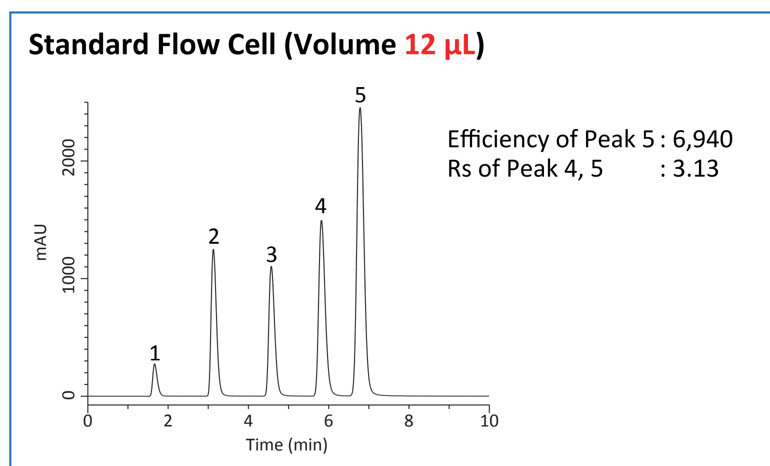


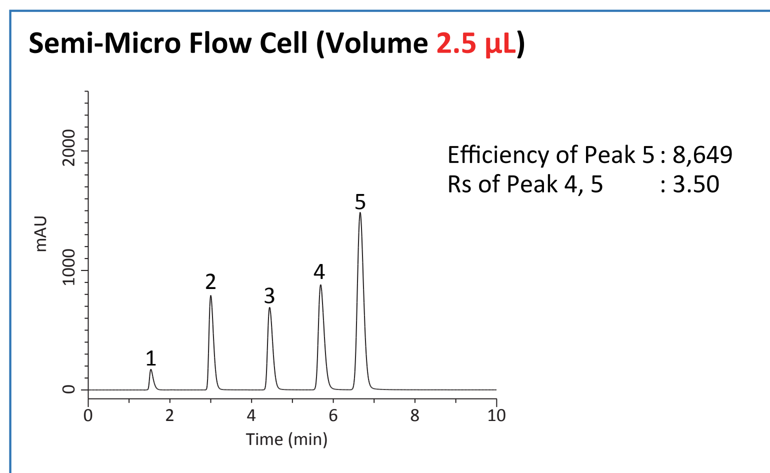
7-5 Detector Flow Cell Volume

When scaling down to a column with a smaller internal diameter, sample diffusion may occur, not only within the tubing and the column, but at the detector flow cell. Such diffusion can influence the chromatographic results, and so, when using narrower columns, it is important to also use smaller volume flow cells.

●Comparison Between Different Volumes of Detector Flow Cell

(Column ID 2.1 mm, Flow Rate at 0.2 mL/min)





HPLC Conditions

Column: InertSustain C18 (2 µm, 150 x 2.1 mm I.D.)
Eluent: CH_3CN/H_2O (65:35, v/v)

Column Temp.: 40 °C
Detection: PDA, 254 nm

Injection Volume: 10 µL

Sample:
1. Uracil
2. Acetophenone
3. Benzene
4. Toluene
5. Naphthalene

If the structure (e.g., optical path length) of the flow cell is the same, a flow cell with lower volumes will deliver better resolution, albeit with decreased sensitivities.

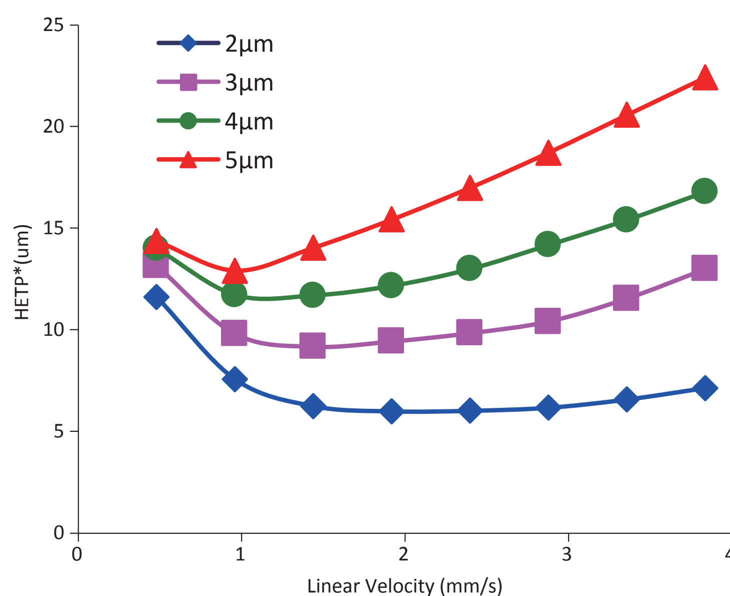
●Comparison of Efficiencies between Column Internal Diameters and Flow Cell Volumes

Column ID (mm)	Standard Flow Cell	Semi-Micro Flow Cell
2.1	6,940	8,649
3.0	10,137	10,713
4.6	19,231	19,195

* The HPLC system was optimized to be used for a 4.6-mm ID analytical column.
* The column length was 150 mm.
* The efficiencies were determined using naphthalene under specific analytical conditions.

8 Tips on Maximizing Efficiency using UHPLC Columns

In HPLC, smaller particle sizes of the packing material will afford higher separation efficiencies. As shown in the following van Deemter plot, smaller particles provide lower HETP* values (higher efficiencies). Furthermore, the lower HETP values are achieved using high linear velocities (high flow rates). However, system optimization (e.g., tubing volume, data sampling rate) is required to maximize the performance of columns with smaller particles. Poor peak shapes and/or resolution may result when using with an unoptimized LC system.



*HETP is an acronym for the Height Equivalent to the Theoretical Plate. It arises from the Plate Theory and is numerically equal to the column length divided by efficiency (the number of theoretical plates) of the column.

8-1 Reduce Extra-Column Volume

●Tubing Volume

Select the appropriate tubing for the HPLC system depending on the internal diameter of the analytical column and on the flow rate of the mobile phase. For more details, please see Section 7-4.

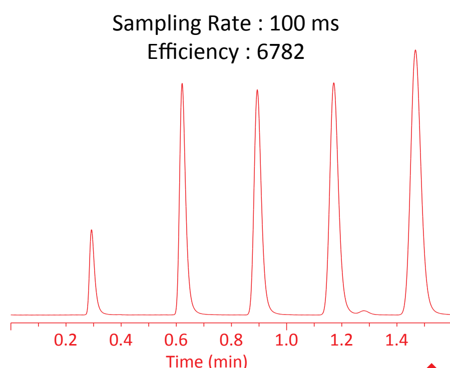
●Detector Flow Cell Volume

Sample diffusion occurring at the detector flow cell can influence chromatographic results. For more details, please see Section 7-5.

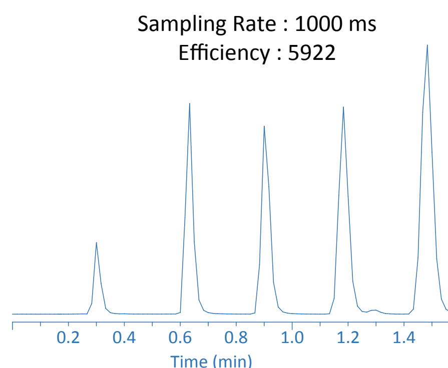
8-2 Data Sampling/Acquisition Rate

The data sampling rate determines the frequency at which detector-signal data points are recorded to construct the chromatogram. Generally, each peak should be defined by at least 20 data points. Insufficient data points may result in wider peaks or irreproducible quantitation results. When using UHPLC columns, the sampling rate should be set at less than 100 ms.

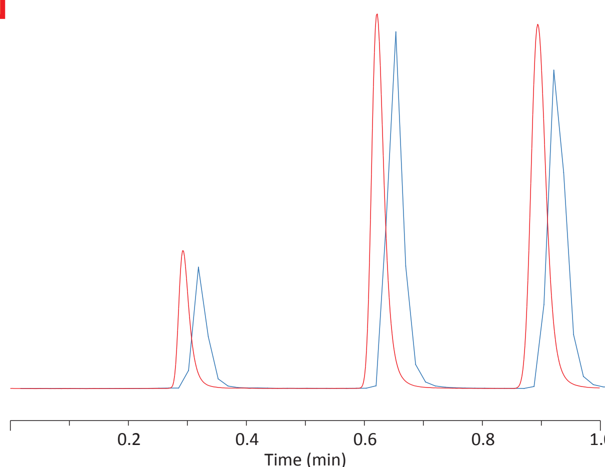
Sampling Rate : 100 ms
Efficiency : 6782



Sampling Rate : 1000 ms
Efficiency : 5922



Enlarged view from 0 to 60 seconds



HPLC Conditions

Column: InertSustain AQ-C18 (1.9 µm, 50 x 2.1 mm I.D.)
Eluent: CH_3CN/H_2O (65:35, v/v)

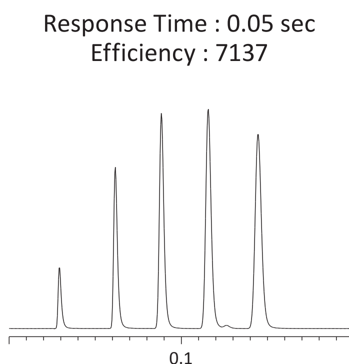
Column Temp.: 40 °C
Detection: UV, 254 nm (Response: 0.1 sec)
Injection Volume: 0.2 µL

Sample:
1. Uracil
2. Acetophenone
3. Benzene
4. Toluene
5. Naphthalene

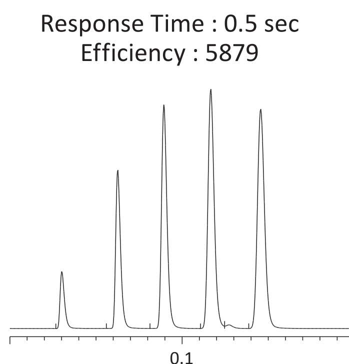
8-3 Response Time/Time-Constant

Response time defines the speed at which a detector tracks sudden changes in the absorbance of the flow cell. To achieve the optimal signal-to-noise ratio, the response time should be set at three times higher than the frequency of the baseline noise. However, response times that are too long can result in broad peaks. Because peak widths are very narrow when using UHPLC columns, shorter response times should be used.

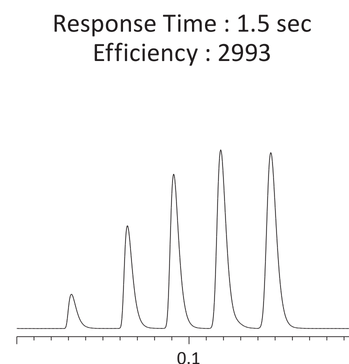
Response Time : 0.05 sec
Efficiency : 7137



Response Time : 0.5 sec
Efficiency : 5879



Response Time : 1.5 sec
Efficiency : 2993



HPLC Conditions

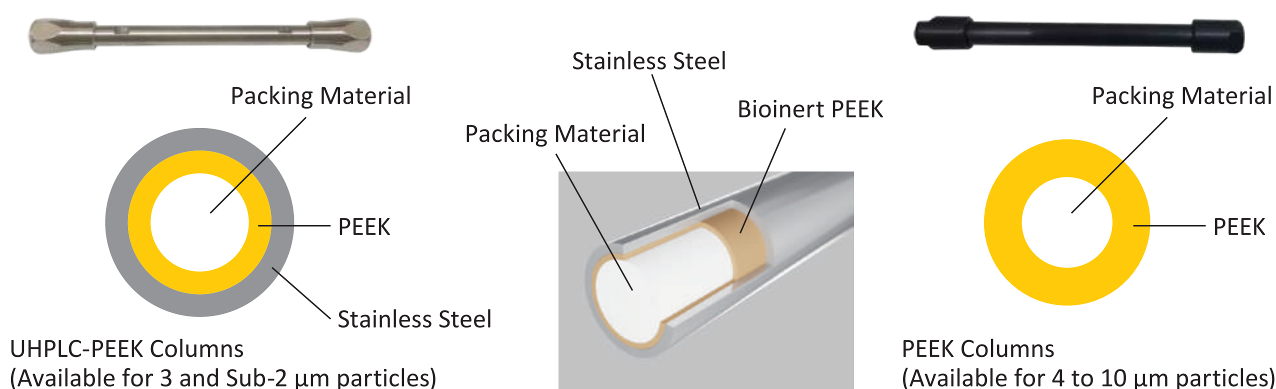
Column: InertSustain AQ-C18 (1.9 µm, 50 x 2.1 mm I.D.)
Eluent: CH_3CN/H_2O (65:35, v/v)
Column Temp.: 40 °C
Detection: UV, 254 nm (Sampling Rate: 100 ms)
Injection Volume: 0.2 µL

Sample:
1. Uracil
2. Acetophenone
3. Benzene
4. Toluene
5. Naphthalene

9 Metal-Free PEEK Columns

9-1 Benefits of Metal-Free PEEK Columns

Analytes containing phosphate groups may form phosphate-iron complexes that often lead to deteriorated peak shapes and/or poor quantitative results. Until recently, it was assumed that residual metallic impurities in the packing material were the cause of such problems. However, recent studies show that the nature of the column hardware may also impact such trace analytes in highly sensitivity analyses. Such interactions can be somewhat mitigated by using mobile phases that contain phosphate buffer or EDTA, which unfortunately are not compatible with LC/MS(/MS).
Alternatively, metal-free polyether ether ketone (PEEK) columns (all wetted parts) are available to minimize unwanted metal interactions, and to improve chromatographic results with better peak shapes and S/N's, especially for bio-chromatographic applications.

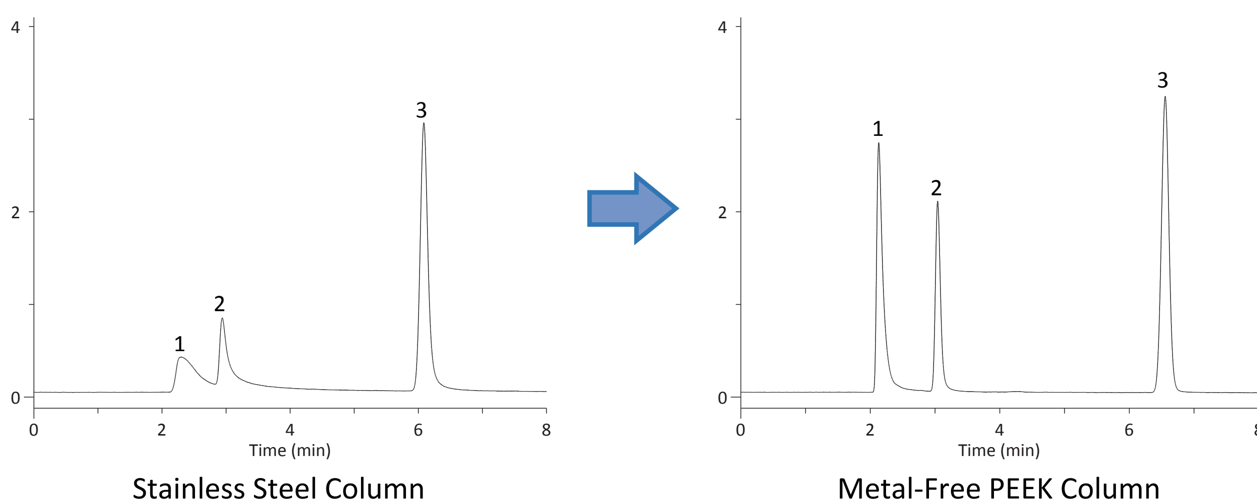


UHPLC-PEEK Columns
(Available for 3 and Sub-2 µm particles)

PEEK Columns
(Available for 4 to 10 µm particles)

●Comparison of Chromatographic Results between Stainless Steel and Metal-Free PEEK Columns

Analytes such as ATP contain phosphate groups, which are highly susceptible to chelating. As shown below, a metal-free PEEK column delivers improved peak shape and S/N compared to a stainless steel column.



Stainless Steel Column

Metal-Free PEEK Column

1. ATP
2. ADP
3. AMP

500 µg/L each

HPLC Conditions

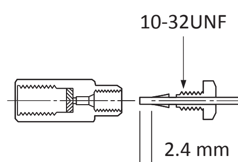
Column: InertSustain AQ-C18 (3 µm, 150 x 4.6 mm I.D.)
Eluent: 5 mM $HCOONH_4$
Column Temp.: 40 °C
Detection: UV, 260 nm
Injection Volume: 10 µL

9-2 Handling of Metal-Free PEEK Columns

Please note the handling of PEEK columns is different than that for stainless steel columns.
Please read the following before using PEEK columns.

- For these columns, all wetted parts are constructed of PEEK. Because the structure of the PEEK column port is especially delicate, do not overtighten the end-fittings. Overtightening the end-fittings may cause damages to the connection parts.

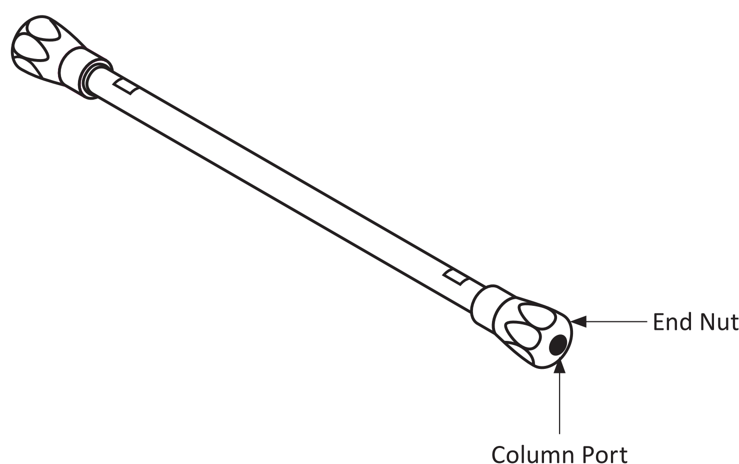
- End-fittings are 1/16” Parker style, UP type.



- Connections to PEEK columns require less strength/force than that required for stainless steel columns. Gently connect the PEEK columns to the tubing, then inspect for any leaks. The torque required for a proper connection is approximately 0.8 N-m.

- Never use worn fittings - always use new fittings. Use of worn fittings may cause damages to the PEEK column port.

- As shown below, when installing or removing the PEEK column, hold the end-fitting and the end nut to tighten or loosen the connection.



- When storing the PEEK column, use the provided column plugs to seal the column. Do not overtighten the column plugs when sealing the column - overtightening the column plugs may cause damages to the connection parts.

- In general, PEEK columns are resistant to a wide range of organic solvents. However, the use of THF or chloroform may damage the surface of PEEK and cause irreparable damage. Avoid the use of THF and chloroform.

10 Tips on Maximizing Purification Efficiencies for Preparative Columns

The ideal properties of the packing materials for preparative separations must include not only the selectivity necessary for optimal separations but also the loadability that enables maximum throughput. In this section, tips on sample loadability and selection of appropriate column dimensions are described.

10-1 Sample Loadability

In preparative HPLC, the goal is to isolate and purify the target analyte. Although the peak shape of target analyte may not look sharp (as compared to optimized analytical conditions), the resolution only needs to be maximized between the target and the nearby peaks.

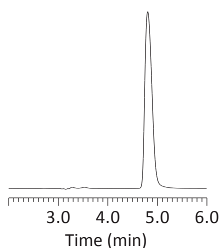
●Step 1: Evaluate the Sample Loadability

- Prepare a sample solution as concentrated as possible.

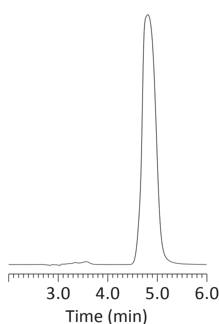
- Inject the sample solution with increments of injection volumes to evaluate the sample loadability. To efficiently evaluate the sample loadability, use injection volumes from 2- to 10-fold).

- With increasing injection volumes, the peak shape will begin to deteriorate. The injection volume at which the peak shape deteriorates would correspond to the maximum sample loadability.

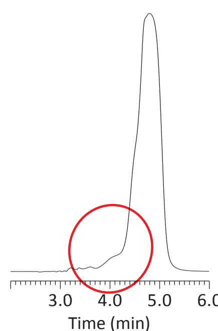
Injection Vol.: 10 µL
(Loadability 1.0 mg)



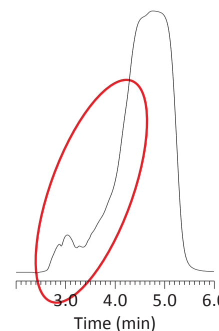
Injection Vol.: 50 µL
(Loadability 5.0 mg)



Injection Vol.: 100 µL
(Loadability 10 mg)



Injection Vol.: 250 µL
(Loadability 25 mg)



Column : Inertsil WP300 C18 (5 µm, 250 x 4.6 mm I.D.)
Eluent : CH_3OH / H_2O (90:10, v/v)
Flow Rat : 1.0 mL/min
Column Temp. : 40 °C
Detection : UV 270 nm
Sample : *tert*-Buthylbenzene (Appox. 10 %, Diluent: Eluent)

As shown above, the peak shape showed deterioration when injecting 100 µL. The peak shape was completely deteriorated and split into two peaks when injecting 250 µL. For this particular method, we can determine that the maximum sample loadability is approximately 100 µL (10 mg).

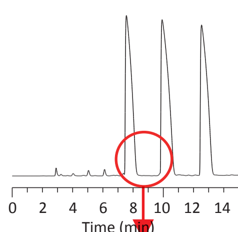
●Step 2: Optimize Separation between the Target Analyte and nearest peaks

•Prepare a sample solution as concentrated as possible.

•Inject the sample solution with small increments of injection volumes to evaluate the resolution. To efficiently evaluate the resolution, use various injection volumes of 2 to 10 fold.

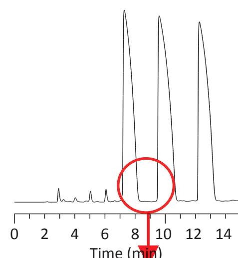
•With increasing injection volumes, the resolution decreases as the separation approaches overload. A resolution factor greater than 1.5 is defined as baseline separation between two neighboring peaks. Depending on the purification criteria, a resolution factor around 1.2 may also be acceptable.

Injection Vol.: 50 µL
(Total Loadability 4.5 mg)



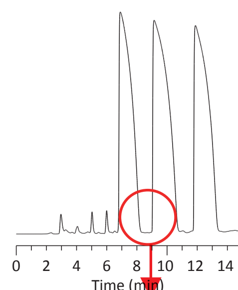
Rs 2.7

Injection Vol.: 100 µL
(Total Loadability 9.0 mg)



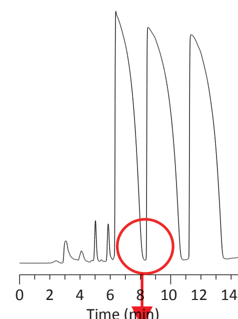
Rs 1.8

Injection Vol.: 200 µL
(Total Loadability 18 mg)



Rs 1.2

Injection Vol.: 400 µL
(Total Loadability 25 mg)



Rs 0.8

Column : InertSustain C18 (5 µm, 250 x 4.6 mm I.D.)
Eluent : CH_3OH / H_2O = 60/40 – 15 min – 100/0, v/v
Flow Rate : 1.0 mL/min
Column Temp. : 40 °C
Detection : UV 270 nm
Sample : Alkylphenol C2-C4 (3.0 % each) Diluent: Eluent

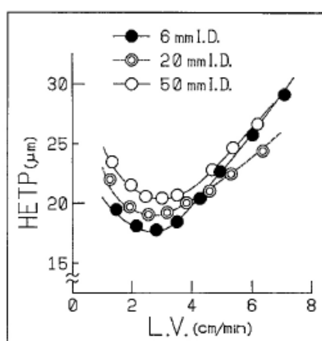
If we were to define the resolution factor of 1.2 as the limit of sample loadability, we can determine the maximum injection volume as approximately 200 µL (total loadability 18 mg) for this particular method.

10-2 Scale-Up

For scale-up applications, a method developed for analytical purposes is directly scaled-up to larger internal diameter columns. An important factor is the ratio of the cross-section between the analytical and preparative columns.

●Step 1: The Flow Rates are Proportional to the Cross-Section of the Columns

As shown below, HETP values were plotted against liner velocity (L.V.) using three columns with different internal diameters, all packed with 10-µm packing material (lower HETP equals higher efficiency).
For all three columns, the optimal linear velocities were at 3.0 cm/min (0.5 mm/sec). Therefore, regardless of the column internal diameter, when using a column packed with 10-µm particles (same bonded phase), the flow rate should be adjusted to a linear velocity of 3.0 cm/min for maximum efficiencies. Linear velocities are calculated by dividing the flow rate by the cross-section of the column. When switching to a column with different diameter, the linear velocity should be kept constant by adjusting the flow rate in proportion to the column cross-section, which is directly proportional to the square of the ratio of the column diameters.



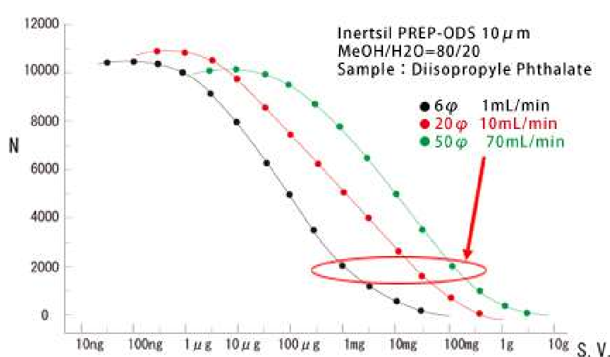
*The optimal linear velocity is dependent on the particle size and the separation mode. For successful scale-up, use the same bonded phase and particle size in both analytical and preparative columns.

●Step 2: The Sample Loadability is Proportional to the Cross-Section of the Column

As shown below, efficiencies (N) versus sample loadabilities (S.V.) were graphed for three columns with different internal diameters. For example, for efficiencies of N=2000, the sample loadabilities for each column are as follow:

ID 6mm : Approx. 1 mg
ID 20mm : Approx. 10 mg
ID 50mm : Approx. 70 mg

For preparative scale-up, injection volumes should also be adjusted in proportion to the column cross-section to achieve equivalent separation efficiencies.



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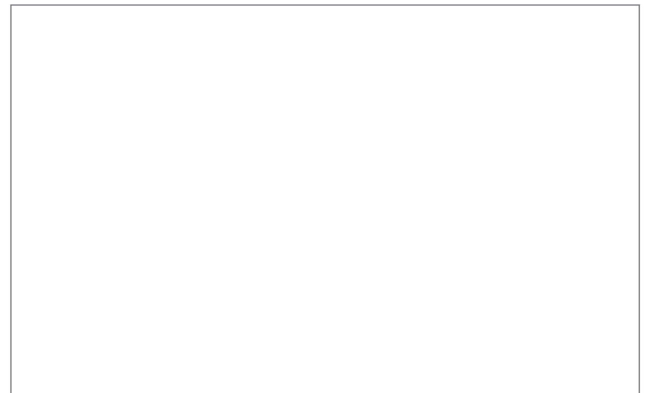

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